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Lost before found?

ON SYSTEMATICS AND CONSERVATION OF LICHEN GENUS *MICAREA* Fr. (Pilocarpaceae, Ascomycota)

Annina Launis

ACADEMIC DISSERTATION

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”The laws of biology are written in the language of diversity.”

O. E. Wilson

List of original articles

The thesis is based on the following articles that are referred to in the text by their Roman numerals:

I. **Launis**, **A**. & Myllys, L. 2014. *Micarea byssacea* new to North America and *Micarea hedlundii* new to Maine, Michigan and Quebec. Opuscula Philolichenum 13, p. 84-90.

II. **Launis**, **A**., Pykälä, J., van den Boom, P., Sérusiaux, E. & Myllys, L. Four new epiphytic species in the *Micarea prasina* group from Europe. Accepted to the Lichenologist.

III. **Launis**, **A**., van den Boom, P., Malíček, J., Sérusiaux, E., Svensson, M., Tsurykau, A. & Myllys, L. Sharpening species boundaries in the *M. prasina* group with notes on the type species *M. prasina*. (Submitted to Mycologia).

IV. **Launis**, **A**., Poczai, P. & Myllys, L. Evolution of wood-dependent species in the *Micarea prasina* group, and a new species *Micarea fennica* sp. nov. on dead wood. (Manuscript).

V. Saine, S., Aakala, T., Purhonen, J., **Launis**, **A**., Tuovila, H., Kosonen, T. & Halme, P. 2017. Effects of local forest continuity on the diversity of fungi on standing dead pines. Forest Ecology and Management 409: 757-765.

Table of contributions

The following table indicates the major contributions of authors to the original articles and manuscripts

	I	II	III	IV	V
Original idea	**AL,** LM	**AL,** LM	**AL,** LM	**AL,** LM	PH, SS
Data collection	**AL**	**AL,** JPy, ES, PB	**AL,** JM, AT, ES, MS, PB	**AL**	SS, HT, **AL,** TA, PH
Morphological data	**AL**	**AL**	**AL**	**AL**	**AL,** HT, TK
Molecular data	–	**AL**	**AL**	**AL**	–
Analyses	**AL**	**AL,** LM	**AL,** LM	**AL,** LM, PP	SS, JPu, PH
Manuscript preparation	**AL,** LM	**AL,** LM, ES, PB	**AL,** LM	**AL**	SS, PH, TA, JPu, HT, TK, **AL**

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JM = Jiří Malíček
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SS = Sonja Saine
TA = Tuomas Aakala
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Contents

Abstract

The lichenized genus *Micarea* Fr. (Ascomycota) comprises ca. 100 species. All species in the genus are crustose and mostly inconspicuous. The species are known from wide range of habitats, and they can cover large surface areas. Many of them are epiphytes on bark or wood, but several are also frequently encountered on rocks or soil. Some of the species are specialized living in old-growth forests or in strict microhabitats, and these species may be sensitive to forest management practices. *Micarea* includes one of the largest groups of congeneric obligate lignicoles depended entirely on dead wood. In addition, many species are regarded as facultative lignicoles.

Micarea prasina group is a monophyletic subgroup within the genus, including the type species of the genus *Micarea prasina* Fr. At the moment, the group includes 28 species. Challenges in species delimitation within this group are often noted. For example, the type species *M. prasina* is known for its phenotypic variability and taxonomic difficulties.

The aim of this thesis was to investigate the deficiently known lichen genus *Micarea* focusing especially on the *M. prasina* group. Moreover, the aim was to gather new information on the distribution, taxonomy, systematics, and evolution of wood-dependency within this group. We also studied the conservation biology of wood dependent communities of *Micarea* and decomposer fungi, and underline the importance of cooperation between taxonomists and ecologists. The specimens for the thesis were collected from several European countries and from the USA. In addition, herbaria collections were examined.

The main results are: 1.) We present a three-loci phylogeny of the *Micarea prasina* group and circumscribe nine new species based on phenotypic characters and phylogenetic analysis. Crystalline granules are studied as a novel character for the species. They are shown highly relevant in linking the old type specimen of *M. prasina* to fresh material. Also, a new species for North America is discovered. 2.) We bring new insights to the evolution of wood-inhabiting *Micarea* species and their reproduction. We suggest that lignicolous substratum requirement has evolved multiple times independently, and that obligate lignicoles are usually anamorphic. 3.) We show that local forest continuity is important for species rich *Micarea* communities. These communities seem to depend on dead standing pine trees that have been available continuously for long periods. However, local continuity did not explain diversity of decomposer fungi.

Our results indicate that species diversity is still rather poorly known even in the relatively well-studied areas of Europe. Understanding species boundaries is a necessity for reliable conclusions in habitat requirements and threat status of the species. Two of the new species described in this study are likely obligate lignicoles occupying strict microhabitats. Intense forest management can pose a real threat to these species.

Summary

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1. INTRODUCTION

1.1 Lichens in the DNA era

Species is a fundamental unit in biology. Nevertheless, species delimitation can be challenging due to many reasons, such as variation of phenotypic characters within and between species (Lumbsch & Leavitt 2011), difficulties in recognizing reliable diagnostic characters and interpreting them (e.g. Nixon & Carpenter 2011; Spribille *et al.* 2011), and even because of the diversity of species concepts (De Queiroz 2007; Grube & Kroken 2000).

Most lichen species are delimited based on similarity or dissimilarity in morphological and anatomical characters. Secondary chemistry has also played a major role in the traditional species delimitation in many lichen groups (Brodo 1978, 1986; Lumbsch 1998). Nowadays, phenotypic characters (including morphology, anatomy and secondary chemistry) are usually integrated with molecular data. Molecular studies on several lichen groups have shown that the true level of diversity has been underestimated and the number of species is higher than previously recognized (Lücking *et al.* 2009; Leavitt *et al.* 2011; Magain *et al.* 2017). For example, molecular studies have revealed a diversity of species with subtle or nonexistent phenotypic differences that are referred to as semicryptic or cryptic (e.g. Bickford *et al.* 2007; Crespo & Pérez-Ortega 2009; Crespo & Lumbsch 2010; Leavitt *et al.* 2011; Leavitt *et al.* 2016; Zhao *et al.* 2017). On the other hand, molecular studies have also been helpful in finding morphological and chemical characters reciprocally (Spribille *et al.* 2011; Truong *et al.* 2013; Spribille *et al.* 2014; Pykälä *et al.* 2017).

Molecular methods have undoubtedly revolutionized the research on species and their evolutionary history. They allow species identification through DNA barcoding and large multi-loci or even genome-wide data sets (e.g. Abrego *et al.* in press; Miadlikowska *et al.* 2006; Schoch *et al.* 2012). Compared to the traditional methods based on phenotypic characters, they are faster and rely less on species specialists with long and costly training. Nevertheless, understanding morphology is still crucial. This is so in describing new species, creating reliable DNA barcodes, and also in linking old specimens such as type material to fresh material (II & III). Morphological characters are also central in practical species identification (I). Therefore insightful research on morphology is still much needed.

Box 1: Phylogenetic systematics

Systematics is the science of studying species diversity, relationships and evolutionary history – and organizing species into hierarchical system. The principle is that classification should reflect the natural relationships, i.e. the phylogenetic relationships (Lipscomb 1998). According to this principle, all taxa should be monophyletic, meaning they should include all descendants from a common ancestor, and no more or less.

Monophyletic groups are characterized by synapomorphies that are unique derived characters shared by all members of the group. These characters can represent evolutionary new morphological, molecular or ecological traits (Wenzel 2002). All groups are not monophyletic, though, but paraphyletic and polyphyletic groups are also recognized. A paraphyletic group includes descendants from a common ancestor, but in contrary to monophyletic group, one or more of the descendants are excluded to form separate groups. A polyphyletic group, on the other hand, is a group that includes unrelated taxa that do not share a common ancestor. These taxa share similar, but in fact, independently evolved characters called homoplasies (Nixon & Carpenter 2011). Homoplasies can be seen as errors in classification.

1.2 *Micarea* – a widespread but poorly known genus

The lichen genus *Micarea* Fr. comprises c. 100 species (Kirk *et al.* 2008; Coppins 2009). All species in the genus are crustose in growth form and mostly small and inconspicuous. Apothecia, if present, are only ca. 0.5 mm in diameter or less. However, ecologically *Micarea* is far from insignificant as the species often cover large surface areas. Many of the species are epiphytes on bark or wood, and some are obligate lignicoles depending entirely on dead wood. Several species are also frequently encountered on rocks or soil of low pH. Some are specialized living in old-growth forests or in strict microhabitats. These species may be sensitive to forest management practices and are also potentially suitable as indicators of the changes in forest landscapes. The genus is best known and most widely collected from Europe where it is widespread and many of the species are regarded as common (Coppins 1983; Czarnota 2007).

Research on *Micarea* has mostly focused on the species diversity and systematics, and less on the ecology and conservation biology (but see Svensson *et al.* 2013, 2016; and also V). One of the earliest monographic works including *Micarea* spp. was published in 1892 by Hedlund. Later, two significant revisions of the genus focused on European species (Coppins 1983; Czarnota 2007). Recently, systematic studies based on molecular data showed that *Micarea* is a polyphyletic group nested within Pilocarpaceae and Psoraceae (Andersen & Ekman 2005; Sérusiaux *et al.* 2010). The results agree well with the previous sub-groupings by Coppins (1983) based on phenotypic characters. Lately, the polyphyly of *Micarea* has been addressed and a new genus *Brianaria* Ekman & Svensson introduced for the *M. sylvicola* group within Psoraceae (Ekman & Svensson 2014). However, *Micarea* still remains paraphyletic.

The type species of the genus, *Micarea prasina* Fr., is nested within a monophyletic subgroup referred to as the *M. prasina* group. The group consists of 28 species (II–IV) which are mostly epiphytic or epixylic on wood or bark. They are characterized by "micareoid" photobiont (a coccoid green alga with cells 4–7.5 µm in diameter), immarginate small apothecia, hyaline hypothecium, branched paraphyses and an ascus of the *Micarea*-type (Coppins 1983; Hafellner 1984; Czarnota 2007; II). Many of the species form effuse thallus composed of goniocysts and several of the species frequently occur without apothecia but with numerous pycnidia. Many produce the Sedifolia-grey pigment (K+ violet, C+ violet), which is typically present in the pycnidia and in the upper layer of the apothecia (Coppins 1983; Czarnota & Guzow-Krzemińska 2010). The most commonly encountered secondary metabolites are micareic, methoxymicareic and gyrophoric acids. Challenges in species delimitation within the *M. prasina* group are well noted and have been discussed by Coppins (1983), Czarnota (2007), Czarnota & Guzow-Krzemińska (2010) (see also I–III). For example, the type species *M. prasina* is known for its phenotypic variability and taxonomic difficulties.

In spite of the two monographic treatments and other systematic studies, *Micarea* is likely still insufficiently known. New species and distribution data are frequently published from Europe (Czarnota & Guzow-Krzemińska 2010; Svensson & Thor 2011; Guzow-Krzemińska *et al.* 2016; Boom *et al.* 2017; II–IV), as well as from other less known areas (Cácares *et al.* 2013; Aptroot & Cácares 2014; van den Boom & Ertz 2014; Brand *et al.* 2014; Córdova-Chávez *et al.* 2014; McCarthy & Elix 2016; I).

1.3 Wood-inhabiting species

Dead wood is crucially important for forest biodiversity as decaying trees offer habitats for thousands of species (Siitonen 2001; Stokland *et al.* 2012). In the Nordic countries, over 7500 species are dependent on dead wood (Stokland & Meyke 2008). In areas of forest management, however, the environment for wood-inhabiting species has altered dramatically: it is estimated that up to 97–98 % of dead wood is removed from managed forests (Fridman & Walheim 2000), and wood based biofuel industry is still increasing the demand (Walmsley & Godbold 2010). In Finland, diminishing amount of dead wood affects approximately every 10th species and is ranked as one of the most common reasons for forest species endangerment (Rassi *et al.* 2010). Similar decline in wood-inhabiting species is also recorded elsewhere (Angelstam 1997; Linder & Östlund 1998; Wilhere 2003; Stokland *et al.* 2012). Furthermore, wood-inhabiting fungi are among the organism groups suffering most from forest fragmentation (Nordén *et al.* 2014; Flensted *et al.* 2016).

Dead wood is a diverse and usually fast changing ephemeral substratum. Logs, snags and stumps provide a number of distinct habitats that differ in many characteristics such as in the amount of light and moisture (Stokland *et al.* 2012; Fig. 1). Different stages of decay may also affect species composition: Lõhmus & Lõhmus (2001) showed that lichen flora of standing trees changes from when the tree is alive, to tree death and loss of bark, until the tree finally falls. The diversity of dead wood also differs on landscape level between managed and natural

Fig. 1. Diversity of dead wood in Evo Nature Reserve, Southern Finland. Photo: Annina Launis.

forests. In natural forests, the diversity and volume of dead wood is higher, both in space and time, better maintaining the continuity of resource availability for wood-inhabiting species. The dramatic decline of natural forests is therefore alarming. Especially vulnerable are species occupying strict microhabitats in natural and old-growth forests (Fritz *et al.* 2008; Stokland *et al.* 2012; IV & V). In species conservation, the continuity of suitable habitats for species should be viewed in landscape and local level (Fritz *et al.* 2008; Hanski 2005; Nordén *et al.* 2014).

In their comprehensive review mainly based on herbarium material, Spribille *et al.* (2008) found that as much as 550 lichen species occupy dead wood in Fennoscandia and Pacific Northwest of North America. Of these, 132 species are obligate lignicoles not known from other substrata and 418 are facultative lignicoles that occupy dead wood but also other substrata such as bark of living trees or rock and soil. Most of the obligate lichen species are poorly known microlichens. *Micarea* includes one of the largest groups of congeneric obligate lignicoles and in addition, many other *Micarea* species are regarded as facultative lignicoles (Coppins 1983; Czarnota 2007; Spribille *et al.* 2008). Wood-dependency is common within the *M. prasina* group, where seven species are regarded as obligate lignicoles (Czarnota 2007; Spribille *et al.*

2008; Guzow-Kremínska *et al.* 2016; III & IV), and 11 species are facultative lignicoles (Coppins 1983; Czarnota 2007; Czarnota & Guzow-Krzemińska 2010; van den Boom *et al.* 2017; I–III). Some of the facultative species are encountered often on lignum while others occur very rarely on the substratum (IV).

1.4 From species to communities

Recent studies have shown that majority of the wood-inhabiting fungi are small-sized non-lichenized or lichenized Ascomycetes (Spribille *et al.* 2008; Rämä *et al.* 2014). However, such species are often poorly known and therefore their taxonomy, systematics, species interactions and the diversity within and between communities need scientific attention. While understanding species boundaries can be considered important and interesting *per se*, the correct taxonomy is also a necessity for reliable conclusions to be made concerning habitat requirements and threat status of the species. In community studies species delimitation is also crucial. However, modern studies on community ecology rarely discuss on how the studied species were identified, nor use appropriate taxonomic literature or expertise of trained taxonomists (Bortolus 2008). Species misidentifications can lead to major errors in ecological studies and even affect our understanding of nature.

Research on the communities of small-sized species is faced with many challenges. Data collection and identification is very time consuming, and success relies on active cooperation between scientific fields. But as mentioned earlier, traditionally ecologists and taxonomists have not collaborated sufficiently. Ecologists tend to use the well-known species groups as their model organisms, and many ecological studies on dead wood have actually excluded the large diversity of small-sized fungal species (Heilmann-Clausen 2001, Abrego & Salcedo 2013). Taxonomists, on the other hand, explore the poorly known diversity and often specialize on one or only a few groups. For these reasons, active communication and cooperation between the fields would likely benefit the researchers and data collection, not to mention induce more reliable and applicable results (Halme *et al.* 2015). In *Micarea*, this kind of approach has not been conducted before.

2. AIMS AND OUTLINE OF THE THESIS

The main aim of this thesis is to investigate the deficiently known lichen genus *Micarea* focusing especially on the *Micarea prasina* group. We bring new information on the distribution (Chapter I), taxonomy and systematics (Chapters II–IV), evolution of wood-dependency (Chapter IV), and conservation biology (Chapter V) of the species and communities.

The specific aim of Chapter I is to study occurrence and distribution of *Micarea byssacea, M. hedlundii* and the allied species in North America. According to Esslinger (2014), 40 *Micarea* species are known from North America, of which eight belong to the *M. prasina* group. Many of the species are poorly known and under collected.

Chapter II studies the phylogenetic relationships and reassess the current taxonomy of the *M. prasina* group focusing especially on the *M. byssacea* and *M. micrococca* complexes. The aims are to unveil the undescribed diversity within the group, to clarify species boundaries and to examine the distribution and ecological requirements of the species. Because of small amount of distinct phenotypic characters in the groups – causing obvious challenges in species delimitation – the crystalline granules were studied as a novel phenotypic character.

Chapter III continues to explore the species diversity and phylogenetic relationships in the *M. prasina* group. The work focuses especially on the *M. prasina* complex, i.e. a subgroup within the *M. prasina* group. This species complex includes the type species of the genus, *M. prasina*. Previous studies have shown that *M. prasina* is morphologically variable (Coppins 1983; Czarnota 2007) and infraspecific genetic variation between European and North American specimens has been reported (e.g. Czarnota & Guzow-Krzemińska 2010). Our study also discusses challenges concerning type specimens that are too old for molecular identification. We further examined the use of the crystalline granules as a novel phentotypic character in species delimitation within *Micarea*.

Chapter IV investigates the phylogeny and the evolution of wood-dependent species in the *M. prasina* group. The aim is to study the character evolution of wood-dependency and reproduction. In addition, the undescribed diversity within the group is further explored.

Chapter V aims to show how differences in local forest continuity (both in stand and microhabitat level) effects the diversity of fungi on dead standing pines. Previous studies have shown the importance of landscape-level continuity to the wood-inhabiting fungi (Flensted *et al.* 2016; Gu *et al.* 2002; Junninen & Komonen 2011; Paltto *et al.* 2006; Ranius *et al.* 2008; Sverdrup-Thygeson & Lindenmayer 2003), but the effects of local continuity has remained controversial (Fritz *et al.* 2008; Groven *et al.* 2002; Rolstad *et al.* 2004; Svensson *et al.* 2013; Sverdrup-Thygeson & Lindenmayes 2003). The species richness and community composition is studied on *Micarea* and non-lichenized decomposers. The effects of variables, such as past management intensity, availability and diversity of dead wood in the area, and age of the studied stumps are investigated. The investigated groups of fungi differ in their nutrition biology and dispersal ecology opening rare possibilities for exploring whether the effects of local continuity are different for different fungal groups.

3. MATERIAL AND METHODS

3.1 Material

The specimens for the thesis were collected by the author or received from colleagues from several European countries and from the USA. In addition, dried lichen specimens of various ages were obtained from several herbaria and private collections. For Chapters I–IV the lichen specimens were mostly collected by randomly searching from managed, old-growth or natural forests. The specimens were searched for and collected from several tree species. Both living and dead trees were studied. Dead wood of various ages, decay classes and both standing and fallen were inventoried. For Chapter V, on the other hand, lichens and decomposer fungi were collected only from dead standing *Pinus sylvestris* trees selected along a 10-m wide transect.

More specifically, for Chapter I the material was collected during a field excursion in 2012 to North America, Maine, by the author of the thesis. In addition, collections were obtained from several North American and European herbaria (CANL, H, NBM, O) for *M. hedlundii*.

For Chapters II–IV, the specimens were collected from Finland (by the author of the thesis and Dr. Pykälä), Belarus (Dr. Tsurykau), Czech Republic (Dr. Malíček), France (Dr. Sérusiaux), the Netherlands (Dr. Brand, Dr. van den Boom, Dr. Sérusiaux), Scotland (the author together with Dr. Coppins), Sweden (the author and Dr. Svensson), and the USA (the author) during 2002–2015. Additionally, sequences of several specimens were obtained from GenBank. Type material of the related *Micarea* taxa from the herbaria G, H, and UPS were studied for comparison.

For Chapter V, the specimens were collected from 14 study forests located in central Finland. The specimens were collected by the author of the thesis (*Micarea* lichens), Dr. Tuovila (Mycocaliciales) and MSc. Saine (agarics, corticioids, discomycetes, jelly fungi, polypores and pyrenomycetes). The trunks were carefully examined throughout from ground level up to a height of 1.8 meters. Species of Mycocaliciales were recorded only from sapwood whereas all other fungal groups were examined also from bark. The specimens were identified by species experts of each groups. Several variables were recorded in the field for each study trunk. These included coordinates, circumference at breast height (cm), height (m), decay stage (1–5) estimated by knife (Renvall 1995), the proportion of surface not covered by bark (%) and the coverage of lichens (%).

3.2 Morphology and chemistry

In Chapters I–V, the morphological examinations of the *Micarea* specimens were carried out as follows. Hand cut sections of apothecia or squashed preparations of pycnidia and thalli were examined with a dissecting or compound microscope. Ascospore dimensions and other anatomical measurements were made in water and usually further examined in 10 % potassium hydroxide (K). Identification followed Coppins (1983), Czarnota (2007), and Czarnota & Guzow-Krzemińska (2010). The crystalline granules were investigated by using compound microscope with polarization lenses. In Chapter V, decomposer fungi were identified at species level in the field if possible. Otherwise, specimens were collected for later microscopic identification

in the laboratory. Species nomenclature follows Index Fungorum (Royal Botanic Gardens Kew *et al.*, 2016) and for Mycocaliciales Tibell (1999).

For the *Micarea* spp., chemical spot tests were performed under a compound microscope using sodium hypochlorite (C) and 10 % potassium hydroxide (K) (Orange *et al.* 2010). Pigments were defined following Coppins (1983), Meyer & Printzen (2000) and Czarnota (2007). Specimens were further studied using thin-layer chromatography (solvent C) following Culberson and Kristinsson (1970) and Orange *et al.* (2010).

3.3 DNA extraction and sequencing

For Chapters II–IV, DNA was extracted from apothecia, pycnidia or thalli of max. three years old specimens. DNA was extracted using DNeasy® Blood & Tissue kit by Qiagen following the protocol described in Myllys *et al.* (2011). Three DNA regions (ITS, mtSSU and partial *Mcm7*) were amplified and sequenced (Table 1). A total of 176 sequences were generated. PCR reactions were prepared as described in each chapter of the thesis by using PuReTaq Ready-To-Go PCR beads (GE Healthcare). PCR products were cleaned and sequenced by Macrogen Inc., South Korea (www.macrogen.com).

The internal transcribed spacer region rDNA (ITS1-5.8S-ITS2) is a nucleic spacer sequence situated between genes coding the ribosomal small-subunit (18S) and large-subunit (28S). Evolution in the non-coding ITS regions is rapid due to relatively low evolutionary pressure. The ITS regions are widely used in molecular phylogenetics, and also as a DNA barcode for fungi (e.g. Hillis & Dixon 1991; Schoch *et al.* 2012).

The mitochondrial small subunit rDNA (mtSSU) is widely used in the phylogenetic and species-level studies of *Micarea* (e.g. Andersen & Ekman 2005; Czarnota & Guzow-Krzemińska 2010; Sérusiaux *et al.* 2010; Guzow-Krzemińska *et al.* 2016; Boom *et al.* 2017). Evolution is relatively rapid in this region due to low repair mechanisms in the mitochondria (Hillis & Dixon 1991).

The replication licensing factor *Mcm7*-gene encodes a protein that is required for DNA replication initiation and cell proliferation. The region is a highly conserved single-copy gene

Table 1. Primers used in DNA amplification and sequencing.

DNA region	Primer	Sequence 5´ – 3´	Reference	Thesis chapter
ITS	ITS1–LM	GAACCTGCGGAAGGATCATT	Myllys *et al.* 1999	II, III, IV
ITS	ITS4	TCCTCCGCTTATTGATATGC	White *et al.* 1990	II, III, IV
mtSSU	mrSSU1	AGCAGTGAGGAATATTGGTC	Zoller *et al.* 1999	II, III, IV
mtSSU	mrSSU3R	CCCGATATCTGCACGGTGTA	Zoller *et al.* 1999	II, III, IV
Mcm7	*Mcm7*_AL1r	CKGTCACARCSAAGCARTAYACACCTATG	Launis *et al.*: Chapter II	II, III, IV
Mcm7	*Mcm7*_AL2f	CTTTYGTCACWCCSCCRATKAGRAGC	Launis *et al.*: Chapter II	II, III, IV
Mcm7	x.*Mcm7*.f	CGTACACYTGTGATSGATGTG	Leavitt *et al.* 2011b	II, III
Mcm7	*Mcm7*.1348R	GAYTTDGCIACICCIGGRTCWCCCAT	Schmitt *et al.* 2009	II, III

(Kearsey & Labib 1998; Schmitt *et al.* 2009). Before this study, it has not been used in the phylogenetic studies of *Micarea*.

For PCR amplification and sequencing, we mostly used primers obtained from previous studies. For the *Mcm7* gene region a new pair of primers (*Mcm7*_AL1r and *Mcm7*_AL2f) was designed using freely available web services. ThermoFishers Tm Calculator was used to calculate the melting temperatures of the primers (www.thermofisher.com). Net primer – Free web based tool was used to further analyze the primers and their compatibility (www.premierbiosoft.com). The primers were manufactured by Metabion International AG (www.metabion.com).

3.4 Phylogenetic analyses

Phylogenetic relationships can be reconstructed by several methods. Mostly used are methods based on maximum parsimony, maximum likelihood or Bayesian inference. Although all these methods are inferred from different philosophies and based on different optimality criteria, they share in common the idea of trying to reconstruct phylogenetic relationships based on minimum of character transformations (Nixon & Carpenter 2011). In molecular data, each nucleotide is a character with four possible character states: adenine (A), cytosine (C), guanine (G) and thymine (T). The possible transformations are transitions, transversions and nucleotide insertions or deletions. Conducting different methods for reconstructing phylogenetic relationships is advisable: this can open new perspectives to the data, show conflicts in the phylogenetic signal and increase reliability of the results.

In this study, the phylogenetic relationships were reconstructed based on molecular data obtained from three DNA regions (ITS, mtSSU and partial *Mcm7*). Sequences were aligned with MUSCLE v.3.8.31 (Edgar 2004) using EMBL-EBI's freely available webservice (http://www.ebi.ac.uk/Tools/msa/muscle/). The sequence matrices were combined into a concatenated matrix in MacClade 4.08 (Maddison & Maddison 2005). Portions of the alignment with ambiguous positions that might not have been homologous were excluded.

The concatenated data set was subjected to maximum parsimony and maximum likelihood analyses. Maximum parsimony analyses were conducted with the program TNT v.1.1 (Goloboff *et al.* 2008). Maximum likelihood analyses were performed using the program RAxML v.8.1.15 (Stamatakis 2014) located at CSC-IT Center for Science (http://www.csc.fi/english) internet server. GTRGAMMA model was used for all partitions.

3.5 Ancestral state reconstruction

In Chapter IV, the evolution of obligate lignicoles was investigated by reconstructing ancestral states. A binary matrix was prepared with character states given for each taxon (obligate lignicole: yes/no). The reconstructions were made with Mesquite 3.40 (Maddison & Maddison 2018) using parsimony and maximum likelihood methods. The analyses were conducted by Dr. Poczai.

In addition, substratum requirement and reproduction mode were studied by mapping states at the tips of the tree. Substratum requirement was mapped as: 1. obligate lignicole; 2. facultative lignicole; 3. neither. Reproduction mode was mapped as: 1. occurs frequently with numerous mesopycnidia, but without apothecia, or sterile; 2. apothecia usually present and often abundant.

3.6 Microhabitat and stand continuity

In Chapter V, local continuity was investigated at a microhabitat and stand level. The methods were mostly conducted by the first author of the article, MSc. Saine.

The **microhabitat** continuity was studied by using dendrochronological methods to assess the age and time since tree death. A cross-sectional sample disc, or a part of a disc, was extracted from each of the 70 study trunks. The sample discs were studied using visual cross-dating (Yamaguchi 1991) against the increment cores obtained from the living trees. WinDENDRO (Regent Instruments Inc., 2015) was used in measuring the widths of tree rings. Furthermore, COFECHA-software (Holmes 1983) was used in statistically confirming the results of the visual cross-dating. In some of the samples a pith of the tree was missing, and a pith locator (Speer 2010) was needed for estimating the number of missing rings. In addition to cross-sectional sample discs, a master chronology of the study area was built by extracting increment cores from five living trees nearby each study trunk.

Assessing the age of the study trunk at death, was calculated as a difference between the calendar year of the last ring and the pith year. Investigating the time since tree death was, on the other hand, calculated as the difference between the sampling year (2015) and the cross-dated year of the last ring.

The **stand** level continuity was studied by assessing past management intensity from the number of stumps, and by also assessing dead wood diversity. The cut stumps and the diversity of dead wood were studied in four 10 m x 50 m transects located in principal compass points around each study trunk. The number of cut stumps were calculated as the sum of stumps recorded from all transects (transect area being 1 ha). Furthermore, the diversity of dead wood was estimated from pieces of dead pines. All pieces with a diameter minimum of 10 cm were recorded, as were also all fallen and standing dead wood with length or height more than 1 m. In addition to the measurements of diameters, decay stage was estimated for standing and fallen trees.

Based on the measurements of all pieces of fallen and standing dead wood, volumes were calculated using the formula for truncated cone volume. The total dead wood volume of each site was calculated as a sum of volumes of all dead wood within the four transects. Finally, the stand continuity was described by using Shannon´s diversity index (Shannon & Weaver 1949).

3.7 Statistical methods

In Chapter V, local continuity was studied using several environmental variables: stand continuity was explained by dead wood diversity and management intensity, and furthermore, microhabitat continuity was explained by age and time since tree death. Diameter and canopy openness were also used as variables. All statistical analyses were performed separately for *Micarea* lichens and decomposer fungi. The analyses were conducted at trunk level and they were performed using R (version 3.3.2; R Core Team, 2016). The analyses were mostly executed by MSc. Saine and Dr. Halme.

To explore which environmental variables best explain species richness of wood-inhabiting fungi a Generalized Linear Mixed Model (GLMM, n = 52) with a Poisson distribution and a log-linear link function was used (function ”glmer” from the package ”lme4” by Bates *et al.*, 2016). Furthermore, in the models the trunks were treated as nested within sites. The lowest AIC value determined the choice of a model.

To investigate the effects of environmental variables on the community composition, the data was analyzed by using Bioenv (function ”bioenv” from the package ”vegan” by Oksanen *et al.*, 2017). To enable comparisons between pairs of communities all species with only one occurrence and trunks with only one species were excluded from the analysis. To further visualize the effects of environmental variables on the community composition, a Nonmetric Multidimensional Scaling (NMDS) with binary Bray-Curtis dissimilarities (function ”metaMDS” from ”vegan”) were used.

4. MAIN RESULTS AND DISCUSSION

The main results of this thesis are:

A three-loci phylogeny of the *Micarea prasina* group and nine new species descriptions based on phenotypic characters and phylogenetic analysis (II–IV, Table 2, Fig. 2). A new species to North America is also recorded (I).

New insights to the evolution of wood-inhabiting *Micarea* species and their reproduction. Lignicolous substratum requirement is found in several independent lineages across the *M. prasina* group. Obligate lignicoles are predominately anamorphs reproducing by pycnidia and mesoconidia (IV).

Local forest continuity is important for species rich *Micarea* communities, which seem to depend on dead standing pine trees that have been available continuously for long periods. However, local continuity did not explain diversity of decomposer fungi (V).

4.1 Phylogeny of the *Micarea prasina* group including nine new species (I–III)

We investigated the phylogenetic relationships and reassessed the current taxonomy of the *Micarea prasina* group based on morphological and – for the first time – multi-loci molecular data. Altogether 28 species belonging to the *M. prasina* group were analyzed and a total of 176 new sequences were generated. We found three monophyletic subgroups within the *M. prasina* group, i.e. the *M. byssacea* and *M. micrococca* complexes (II), and the *M. prasina* complex (III). Furthermore, we discovered unexpected species diversity within the three complexes (Fig. 2).

We recognized twelve undescribed well-supported lineages within the *M. prasina* group. In this thesis, eight of the lineages are described as new species: *Micarea czarnotae* Launis, van den Boom, Sérus. & Myllys, *M. laeta* Launis & Myllys, *M. microareolata* Launis, Pykälä & Myllys and *M. pseudomicrococca* Launis & Myllys (II), *M. fallax* Launis & Myllys, *M. fennica* Launis & Myllys, *M. flavoleprosa* Launis, Malíček & Sérus. and *M. pusilla* Launis & Myllys (III–IV). The results also support the distinction of *Micarea melanobola* as a species-level taxon. The new species are delimited by morphological and DNA-level characters, and also partly by secondary chemistry and the presence or absence of the Sedifolia-grey pigment (Table 2).

In addition to the new species descriptions, at least three other undescribed well-supported lineages were discovered. One of the lineages is represented only by a single specimen and therefore left undescribed (lineage A, II). Two other lineages are connected to the type species *M. prasina* (III). We found that *M. prasina* is paraphyletic and resolves into three lineages. The phenotypic characters of these lineages were carefully studied for, but for now, we do not propose taxonomic innovations. However, we were able to connect one of the lineages to the original type specimen of *M. prasina* (see below).

Because of the small amount of distinct phenotypic characters in the *M. byssacea*, *M. micrococca* and *M. prasina* complexes, we investigated the potential of the crystalline granules as

a novel character (II–III). The crystalline granules have not been previously investigated in the genus *Micarea,* but have been found important features in some other lichen groups (Brodo 1984; Spribille *et al.* 2011). Our study indicates that the crystals are useful characters in the delimitation of some *Micarea* species. However, more data is needed to better understand the reliability of the new feature within the whole genus. The presence and distribution of the crystals were found unique e.g. in *M. prasina* (granules only in the epihymenium) and in *M. czarnotae* (no crystalline granules in the thallus). Within the *M. byssacea* complex the crystalline features were not found useful, as the size and distribution of these granules were shown to be identical amongst the species. Perhaps most importantly, the crystalline granules were found highly relevant in linking the old type specimen of *M. prasina* to fresh material. The original type specimen was collected in 1825, and hence too old for successful DNA sequencing and subsequent molecular identification.

In Chapter III, we also discussed the benefits and disadvantages of epitypification (III). Challenges in sequencing old type specimens are quite often used as an argument for designing an epitype (Ariyawansa *et al.* 2014). However, at least for now, the nomenclatural code specifically determines that a lack of sequence is not a valid argument for creating an epitype. In the case of *M. prasina*, we successfully connected fresh material with the original well-preserved type specimen by investigating morphology and the crystalline granules. Therefore, in our study, creating an epitype was considered unnecessary.

Studies on the *M. prasina* group also revealed new distribution information for two species in North America (I). *Micarea byssacea* was recorded as new to North America from the coast of Maine, and a rarely collected species *M. hedlundii* was recorded new to Maine, Michigan and Quebec. *Micarea byssacea*, and its close relatives *M. micrococca* and *M. prasina* are likely all common epiphytes in the collection area, but because of their inconspicuousness, morphological resemblance and taxonomic difficulties they are likely overlooked. Further studies are needed to fully understand the ecology and distribution of *M. byssacea*, *M. hedlundii*, and the allied species in North America.

Our results show that species diversity can still be rather poorly known in Europe and the USA. Many of the here described species are probably common – or at least not in any evident threat of extinction. However, this is not always the case. *Micarea fennica* and *M. flavoleprosa* have been collected only from dead wood in old-growth or natural forests (III–IV). The two species are likely obligate lignicoles occupying strict microhabitats. In areas of intense forest management, such as in Fennoscandia and Central Europe, dead wood has become a rare and diminishing substratum. Based on these results, we consider *M. fennica* and *M. flavoleprosa* likely rare and possibly under threat of extinction.

(*Continues on page 22*)

Table 2. The main distinguishing characters for *Micarea byssacea*, *M. micrococca*, *M. prasina* s. str., and the new species described in this thesis.

	Species	Thallus	Thallus colour	Apothecia size (mm) and colour	Asco-spores (µm)	Pycnidia	Pigments in apoth-ecia and pycnidia	Chemistry (TLC)	Other
Micarea byssacea complex	*M. byssacea*	minutely granular, go-niocysts usually fine-ly divided	green to olive green	0.2–0.6 mm; whitish, cream, grey to black-ish grey	(6–) 8.0–12.0 (–13) × 2.7–3.5 (–4.2) µm	micro- and meso-pycnidia, immersed in surrounding gonio-cysts, sometimes sessile	Sedifolia-grey	methoxy-micareic acid	
	M. laeta	goniocysts usually aggregated or form almost continuous crust, if less devel-oped warted	vivid green to olive green	0.3–0.5 (–0.6) mm, whitish or cream white, sometimes brownish	(8–) 8.5–12.0 × 3.0–4.0 µm.	micro- and meso-pycnidia, usually im-mersed in surround-ing goniocysts, some-times sessile	no pig-ments	methoxy-micareic acid	
	M. micro-areolata	goniocysts often co-alescing to form con-vex to subglobose small areolae, often partly granular, if less developed warted	pale olive green, whitish green or bright green	0.3–0.6 (–0.7) mm, whitish cream	7.5–12.0 × (2.0–) 2.2–3.0 µm	micro- and meso-pycnidia, usually im-mersed in surround-ing goniocysts, some-times sessile	no pig-ments	methoxy-micareic acid	
Micarea micrococca complex	*M. czarnotae*	goniocysts often coa-lescing to form dense ± continuous thallus, sometimes cracked, if less developed warted	olive green to darkish olive green	0.1–0.3 mm, cream white or brownish, of-ten with a grey-ish tinge	7.0–10.0 × 2.25–3.5 µm	micro- and mesopy-cnidia, sessile or im-mersed in surrounding goniocysts	Sedifolia-grey	methoxy-micareic acid	no crystal-line gran-ules de-tected in the thal-lus
	M. micro-cocca	minutely granular, go-niocysts usually ag-gregated	bright green to pale olive green	0.1–0.3 mm, whitish, cream	10–12 (–16) × 3.0–4.5 µm	micro- and meso-pycnidia, immersed in surrounding gonio-cysts, sometimes sessile	no pig-ments	methoxy-micareic acid	
	M. pseudo-micrococca	minutely granular, go-niocysts usually ag-gregated	olive green, sometimes partly bright green	0.2–0.3 (–0.4) mm, cream white or often pale brownish	8.0–14.0 (–15) × 2.0–3.2 µm	micro- and meso-pycnidia, immersed in surrounding gonio-cysts	no pig-ments	methoxy-micareic acid	two types of para-physes (up to 2 µm wide)
Micarea prasina complex	*M. fallax*	granular, goniocysts usually aggregat-ed or form ± thick al-most continuous and cracked thallus, if less developed wart-ed or partly membra-nous and ± shiny	vivid green or pale olive green to dark olive green	0.2–0.4 (–0.5) mm, cream white, pale brownish, honey brown to brown, sometimes with greyish tinge	8.0–11.0 × (3.0–) 3.25–4.0 µm	micro- and meso-pycnidia, usually im-mersed in surrounding goniocysts	Sedifolia-grey	micareic acid	
	M. fennica	minutely granular, go-niocysts often aggre-gated	pale green, whitish green, greyish green	unknown	unknown	mesopycnidia numer-ous, stalked with one or up to five borne terminally, simple or branched, dark grey to brownish, covered with thin whitish to-mentum, 0.2–0.5 mm tall (or up to 1 mm)	Sedifolia-grey	micareic acid	
	M. flavo-leprosa	minutely granular or farinose, thick	yellowish-, whitish- to olive green	rare, 0.4–0.6 mm, cream-white	(10–) 12.0–16.0 × 4.0–6.0 (–6.2) µm	micro- and meso-pycnidia, usually im-mersed in surrounding goniocysts	no pig-ments	micareic acid	
	M. melano-bola	granular, goniocysts often aggregated, or if less developed warted	pale to dark vivid green, sometimes olivaceous	0.15–0.4 mm, pale to dark grey or black-ish, sometimes brownish	7.25–11.0 × 2.5–3.75 (–4.0) µm	micro- and meso-pycnidia, usually im-mersed in surrounding goniocysts	Sedifolia-grey	micareic acid	
	M. prasina s. str.	minutely granular, go-niocysts often aggre-gated	whitish green, bright green to olive green	0.2–0.5(–0.8) mm, whitish, greyish, grey-brown to almost blackish-brown or blackish-grey	8.0–12.0 (–14.0) × 3.0–4.2 (–5.0) µm	micro- and meso-pycnidia, usually im-mersed in surround-ing goniocysts, some-times sessile	Sedifolia-grey	micareic acid	crystalline granules always in the epihy-menium, rarely also in the hy-menium
	M. pusilla	minutely granular, thin, goniocysts often aggregated, or if less developed warted or membranous	whitish-green to olive green	(0.07–) 0.1–0.15 (–0.2) mm, white or cream white	7.0–9.0 (–9.5) × 2.0–3.0 µm	micro- and meso-pycnidia, usually im-mersed in surrounding goniocysts	no pig-ments	methoxy-micareic acid	

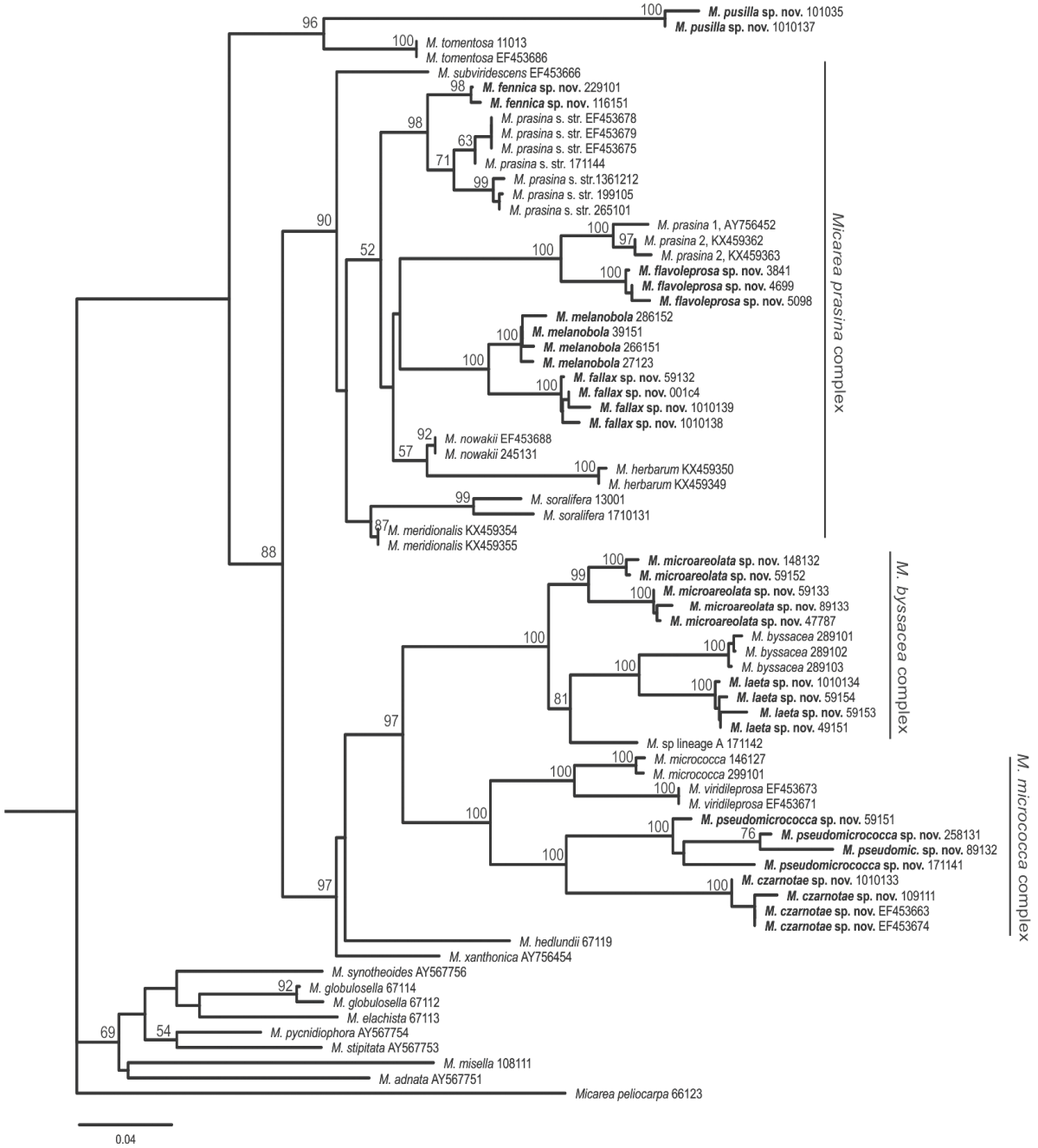

Fig. 2. Phylogenetic reconstruction of the *Micarea prasina* group. New species are indicated in bold (*Micarea czarnotae*, *M. fallax*, *M. fennica*, *M. flavoleprosa*, *M. laeta*, *M. melanobola*, *M. microareolata*, *M. pseudomicrococca* and *M. pusilla*). A maximum likelihood phylogram obtained from RAxML analysis based on the combined ITS, mtSSU and *Mcm7* data set. Branches supported with bootstrap values ≥50% are marked.

4.2 Evolution of wood-inhabiting species in the *Micarea prasina* group (IV)

The *Micarea prasina* group comprises 28 species (II–IV). Based on literature and the results of this thesis, seven of them are regarded as obligate lignicoles (IV). These species are *M. fennica* sp. nov., *M. flavoleprosa* sp. nov., *M. hedlundii*, *M. misella*, *M. nowakii*, *M. soralifera* and *M. tomentosa*. The *M. prasina* group also includes 11 facultative lignicoles some of which are encountered often on lignum, while others are found very rarely on the substratum.

Our results based on the ancestral state reconstruction revealed that obligate substratum requirement is not an ancestral state character in the group, but the obligate lignicoles have evolved multiple times independently across the *M. prasina* group. For example, the newly described obligate lignicoles *M. fennica* and *M. flavoleprosa* are resolved within the *M. prasina* complex. *Micarea hedlundii* is resolved as a separate lineage as a sister to *M. byssacea* and *M. micrococca* complexes, and *M. tomentosa* as a sister to *M. byssacea*, *M. micrococca* and *M. prasina* complexes. *Micarea misella*, on the other hand, is near the base of the phylogenetic reconstruction of the *M. prasina* group. Also the facultative lignicoles are resolved into several lineages within the phylogeny.

The results show rather strikingly that the obligate lignicoles in the *M. prasina* group mostly occur as anamorphs producing primarily pycnidia bearing mesoconidia. The facultative lignicoles and other species in the group mainly reproduce sexually by developing apothecia and ascospores. This phenomenon could be explained by at least two hypotheses: First, species in the *M. prasina* group could be viewed as heterothallic and the rarity of dead wood in space and time could lead into fragmented and geographically isolated populations – Zoller *et al.* (1999 b) showed that this kind of situation could hinder the possibility for finding a mating partner and therefore sexual reproduction would become unnecessary and rare. Plasticity in reproduction of heterothallic species has previously been recorded in several lichen genera (Honegger *et al.* 2004; Honegger & Zippler 2007). A second hypothesis is strategic: species on decaying wood face a significant challenge as their substratum gradually and inevitably vanishes. When this happens, species need to colonize new suitable substrata. Perhaps this sets a time limit where reproduction via mesoconidia, or altogether sterile growth with goniocysts acting as possible diaspores, could be faster and more effective a strategy for successful colonization (Coppins 1983).

Little is still known about the actual roles of the three different types of conidia present in *Micarea* spp., i.e. micro-, meso- and macroconidia. Coppins (1983) suggested that mesoconidia might act as asexual propagules, based on an observation that several species frequently occur without apothecia but have numerous mesoconidia producing pycnidia. This observation is much in line with our result of all obligate lignicoles in the *M. prasina* group producing mesoconidia, or remaining sterile. If mesoconidia are, in fact, asexual propagules they likely act as a fast and energy saving strategy for colonization. Pertinent to this discussion may also be our observation that many primarily mesoconidia producing species occur in shaded situations on fallen tree trunks or on stumps near ground. There they often occur together with sterile *Lepraria* spp. Perhaps in these microhabitats photosynthesis is too low for an abundant production of sexual structures.

4.3 Effects of local forest continuity to communities of fungi on dead pines (V)

Effects of local continuity were studied on species richness and community composition of *Micarea* and decomposer fungi. To explore the effects of environmental variables, our dataset included altogether 107 species of which 91 were decomposer fungi and 16 were species of *Micarea*. Altogether 510 occurrences were recorded.

The results using generalized mixed models show that local forest continuity does not predict decomposer diversity. Diversity of *Micarea*, however, was positively affected by the time since tree death. Environmental variables such as previous management intensity in the site, tree age at death, diameter of the tree or canopy openness did not affect species richness of the decomposer fungi or *Micarea*.

Furthermore, the community composition of decomposer fungi was best explained by dead wood diversity. For *Micarea* on the other hand, the community composition was best explained by the combined effect of years from death, site and dead wood diversity. These results should, though, be viewed with some hesitation as the correlations obtained from the Bioenv analyses were low. In general, the effects of dead wood diversity to community compositions could be explained by shared generalist species. Generalist may be able to survive in areas of homogenous habitat resources and therefore occur rather widely (Norden *et al.* 2013). With higher dead wood diversity the heterogeneity of resources increase and hence these areas can host more diverse species communities, including also specialists (Abrego & Salcedo 2013; Norden *et al.* 2013).

The effects of local continuity can also be inspected at the species level. We found, for example, that the four most common species on the studied stumps were *Micarea melaena*, *Glonium nitidum*, *M. prasina* and *M. misella*. Of these species, occurrences of *Glonium nitidum* were positively affected by canopy openness. *Micarea prasina*, on the other hand, was positively affected by years from tree death. Our study also revealed new information on rarely encountered species. *Micarea eximia*, for example, is previously known only from few collections (Coppins 1983), but was now recorded several times from standing dead pines. I think that the species may well be restricted to this kind of rare habitat as I have never encountered it on any other substratum.

Our results agree well with the previous studies showing restricted or controversial significance of local continuity to wood-inhabiting species (Fritz *et al.* 2008; Groven *et al.* 2002; Rolstad *et al.* 2004; Svensson *et al.* 2013; Sverdrup-Thygeson & Lindenmayes 2003). Although the effects were low for decomposer fungi, species-rich communities of *Micarea* seem to be dependent on standing dead pines that have been available for long time after their death. Therefore, conservation efforts should aim to increase the number of such old trees. Rare specialist species – for example species such as *Micarea fennica* (IV) – might be more sensitive to local continuity, and should be at the center of future research.

5. CONCLUSIONS AND FUTURE DIRECTIONS

Broadening knowledge on the *Micarea* species – their distribution, circumscription, evolution and ecological requirements – has been at the heart of this thesis. This work revealed vast undescribed species diversity within the *M. prasina* group, and furthermore, disentangled taxonomic issues such as the identity of the true *M. prasina* (I–III). We also explored the evolution of wood-inhabiting species and their reproduction (IV), and showed that species-rich communities of *Micarea* are dependent on the continuous availability of standing dead pines over long periods of time (V).

Though DNA methods are central in species identification, insightful research on morphological characters is still much needed. We found that in the *M. prasina* group the previously unstudied crystalline granules were highly relevant in linking the old type specimen of *M. prasina* to fresh material (III). This highlights the importance of thorough anatomical and morphological studies. Lichenologists could even benefit from methods applied in the studies of the non-lichenized Ascomycetes, where knowledge and use of anatomical characters seems to be more diverse.

In the *Micarea prasina* group, obligate lignicoles reproduce mainly by pycnidia and mesoconidia, or remain sterile (IV). The result is very intriguing and could be an indirect confirmation that mesoconidia are, indeed, asexual propagules as the rarity of apothecia rules out a hypothesis that they would be spermatia in the sexual reproduction. Investigating this in a wider scale within *Micarea* or Pilocarpaceae would reveal how common this phenomenon actually is. Species such as *Micarea anterior*, *M. melaeniza, M. nigella* and *M. deminuta* are examples of wood-inhabiting species that do not belong to the *M. prasina* group but, in my experience, commonly reproduce via mesoconidia. Moreover, if thalline goniocysts are soredia-like diaspores, they could enable a safer way for colonization as both the mycobiont and photobiont are present. These aspects require more investigations before we can understand the eco-evolutionary dynamics of these dead wood associated lichens.

High quality taxonomy can certainly benefit research on community and conservation ecology. Our study on the fungal communities on dead standing pines showed how exploring small and deficiently known species can open new range of possibilities and induce unique results (V). Cooperation between taxonomists and ecologists should be valued more, and similar efforts carried out in investigating e.g. fallen logs of different tree species.

All in all, I believe that understanding and sharpening species boundaries has great value in biological sciences. Studies in community ecology, conservation biology and, for example, development of natural history collections depend on it. Especially urgent is to investigate the species occupying strict microhabitats, because such specialists are often rare and may be more sensitive to human induced habitat changes. Such species may be threatened with disappearance before we find them.

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